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Neuroprotection from Stroke in the Absence of MHCI or PirB

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SUMMARY

Recovery from stroke engages mechanisms of neural plasticity. Here we examine a role for MHC class I (MHCI) H2-Kb and H2-Db, as well as PirB receptor. These molecules restrict synaptic plasticity and motor learning in the healthy brain. Stroke elevates neuronal expression not only of H2-Kb and H2-Db, but also of PirB and downstream signaling. KbDb knockout (KO) or PirB KO mice have smaller infarcts and enhanced motor recovery. KO hippocampal organotypic slices, which lack an intact peripheral immune response, have less cell death after in vitro ischemia. In PirB KO mice, corticospinal projections from the motor cortex are enhanced, and the reactive astrocytic response is dampened after MCAO. Thus, molecules that function in the immune system act not only to limit synaptic plasticity in healthy neurons, but also to exacerbate brain injury after ischemia. These results suggest therapies for stroke by targeting MHCI and PirB.

INTRODUCTION

After stroke, the extent of brain and behavioral recovery is influenced by local inflammatory changes and neural circuit plasticity. Inflammation exacerbates damage through a range of mechanisms, including activation of microglia, oxidative stress, and infiltration by peripheral immune cells (Choe et al., 2011; Hurn et al., 2007; Offner et al., 2006). Increased functional recovery is associated with neural plasticity, including axonal sprouting in corticospinal projections that occurs days to weeks after ischemic injury (Carmichael et al., 2001; Lee et al., 2004; Netz et al., 1997). Ischemia induces changes in neuronal excitability and alters dendritic spines within hours (Brown et al., 2007, 2008; Takatsuru et al., 2009). Sprouting and growth of intracortical axons are also thought to serve as substrates for recovery in the somatosensory and visual cortex after peripheral injury or retinal lesion (Florence et al., 1998; Palagina et al., 2009; reviewed in Benowitz and Carmichael, 2010) and can happen rapidly (Yamahachi et al., 2009). On the other hand, cellular correlates of synaptic plasticity, such as long-term potentiation (LTP), are diminished by stroke (Sopala et al., 2000; Wang et al., 2005). These observations suggest that recovery might be enhanced not only by dampening inflammation, but also by increasing synaptic and structural plasticity.

Recently, we discovered that mice lacking major histocompatibility class I (MHCI) function have enhanced visual cortical and hippocampal plasticity not only in development, but also in adulthood (Corriveau et al., 1998; Datwani et al., 2009; Huh et al., 2000; Shatz, 2009). MHCI molecules are expressed in neurons and are located at synapses in the healthy central nervous system (CNS) (Datwani et al., 2009; Needleman et al., 2010), and knocking out (KO) just H2-Kb (Kb) and H2-Db (Db) (KbDb KO), two of the more than 50 MHCI genes, is sufficient to enhance plasticity in mouse visual cortex (Datwani et al., 2009) and cerebellum (McConnell et al., 2009). An innate immune receptor, PirB (paired immunoglobulin-like receptor B) is known to bind MHCI both in neurons (Syken et al., 2006) and in the immune system (Matsushita et al., 2011; Takai, 2005). Like Kb and Db, PirB is expressed in forebrain neurons, and PirB KO mice also have greater visual cortical plasticity (Syken et al., 2006). MHCI molecules are upregulated by inflammation both in the immune system and in the CNS after damage or viral infection (Piehl and Lidman, 2001; Thams et al., 2008). Because these molecules play a dual role in the peripheral immune response and in neural plasticity in the CNS, they could be involved not only in the acute phases of stroke, but also in subsequent recovery. After stroke, these molecules might make a dual contribution to exacerbate damage in the context of the inflammatory response and to restrict recovery by limiting plasticity. Here, we investigate these possibilities by examining response to in vivo and in vitro models of stroke in PirB KO mice and KbDb KO mice.

RESULTS

KbDb KO Mice Recover Better than WT after Stroke

To examine whether Kb and Db contribute to damage after stroke, we gave adult KbDb KO mice (Vugmeyster et al., 1998) transient middle cerebral artery occlusion (MCAO; Han et al., 2009). KbDb KO mice subjected to MCAO had no significant difference from wild-type (WT) in infarct area at 24 hr postinjury

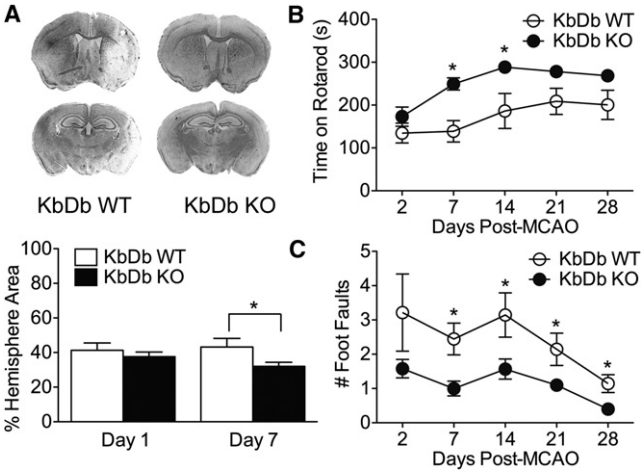

Figure 1. KbDb KO Mice Recover Better than WT Cohorts after MCAO

(A) Top: example of sections of cresyl-violet-stained brains 7 days post-MCAO. Bottom: KO animals have significantly smaller infarct areas at 7 days ($p = 0.03$), but not 1 day ($p = 0.45$), post-MCAO. (B and C) KO animals outperform WT cohorts on motor tasks post-MCAO. Average rotarod (B) and foot fault (C) performances were assessed on days 2 and 7 post-MCAO in all animals, and cohorts of each genotype were kept through 1 month survival, with additional testing on days 14, 21, and 28. p values for the comparisons between genotypes on rotarod on days 21 and 28 were 0.056 and 0.057, respectively, reflecting the reduced numbers of surviving animals. Groups were 19–25 mice (days 2 and 7) and 7–10 mice (days 14–28). *$p < 0.05$. Data are represented as mean ± SEM. See also Figure S1.

(37% versus 41%; $p = 0.45$; Figure 1A), and their initial neurological deficit was also similar ($p = 0.4$; see Figure S1A available online; Han et al., 2009). However, by 7 days post-MCAO, infarct area in KbDb KO mice was modestly reduced (32%) compared to WT (44%; $p = 0.03$). Physiological parameters monitored during surgery were similar between WT and KO and fell within previously reported ranges (Table S1; Han et al., 2009).

To examine motor recovery after MCAO, we tested KbDb KO and WT mice on two motor performance tasks, rotarod and foot fault. Prior to MCAO, KO and WT mice learned both tasks, improving performance over subsequent trials, as evidenced by the increased latency to fall from the rotarod (Figure S1B) and fewer missteps on foot fault (Figure S1C). KO mice learned both behaviors better than WT ($p < 0.001$), consistent with prior observations of enhanced motor learning (McConnell et al., 2009). After stroke, performance on rotarod and foot fault was significantly better in KO mice versus WT ($p < 0.001$ for both paradigms; Figures 1B and 1C). Overall, KbDb KO mice had smaller infarcts and recovered significantly faster and to a greater extent on motor performance (to 91% of prestroke rotarod time compared to 75% for WT at 28 days).

Kb and Db Expression Increase after MCAO

The observations that KbDb KO mice have smaller infarct areas and better behavioral recovery after MCAO suggest that Kb and Db may contribute to damage in WT mice. Moreover, because mice lacking Kb and Db have enhanced synaptic plasticity, it is conceivable that increased expression would contribute to diminished plasticity, thereby compromising recovery. To examine this idea further, we assessed MHCI levels after MCAO. Quantitative real-time PCR (qRT-PCR) revealed highly increased Kb and Db mRNA in the damaged hemisphere (ipsi) compared to sham control after MCAO (Figure 2A) both at 24 hr (Kb mRNA: 2.5-fold increase, $p < 0.05$; Db mRNA: 3.1-fold increase, $p < 0.001$) and at 7 days (Kb mRNA: 8.0-fold increase, $p < 0.01$; Db mRNA: 7.0-fold increase, $p < 0.001$). Kb and Db levels in the damaged hemisphere were also over 2-fold higher than levels in the undamaged hemisphere at 24 hr post-MCAO and over 5-fold higher 7 days post-MCAO (Figure 1A; $p < 0.01$). Western blot analysis of Kb expression in both synaptosome-enriched samples or synaptoneurosomes demonstrated increased protein levels after MCAO in the damaged hemisphere relative to the undamaged side or sham (Figures 2B and 2C).

Because synaptoneurosomes enrich for synaptic proteins (Johnson et al., 1997) after MCAO, Kb protein could be upregulated at synapses and also possibly within glial processes that enwrap the synapse. Previous studies have shown that MHCI proteins are expressed in neurons and are closely associated with synaptic markers in the healthy brain (Datwani et al., 2009; Goddard et al., 2007). MHCI immunostaining, using an antibody known to recognize both Kb and Db (McConnell et al., 2009; Needleman et al., 2010), is primarily associated with neurons in brain sections taken from the cortical penumbra 7 days post-MCAO or from the unmanipulated cortex, as assessed by colocalization with the neuronal marker neuron-specific enolase (NSE). Staining is not detected in astrocytes or microglia (Figures 2D and 2E; Figure S2). As expected, there is evidence of both astrocytic and microglial activation post-MCAO (Figure 2E). Together, these observations demonstrate that Kb and Db are upregulated after MCAO and that within the cortical penumbra, this upregulation is associated with increased protein expression in neurons and at or near synapses.

Improved Outcomes in Mice Lacking PirB Receptor

To explore further how absence of Kb and Db in the brain might lead to neuroprotection, we next examined mice lacking the MHCI receptor PirB (Shatz, 2009; Syken et al., 2006; Takai, 2005). PirB is expressed in CNS neurons, including pyramidal cells, throughout the cerebral cortex. Seven days post-MCAO, PirB KO mice had smaller infarcts than WT (KO: 18% versus WT: 35%; $p = 0.0001$), even though infarct area was the same at 24 hr post-MCAO (Figure 3A). Between 1 to 7 days post-MCAO, infarct area in PirB KO mice decreased significantly (by 51%), as assessed by cresyl violet staining. Because cresyl violet stains acidic cellular components, particularly polyribosomes (Türeyen et al., 2004), the decrease in infarct area in KO mice may reflect recovery of protein synthesis in stressed cells within the penumbra. In KbDb KO mice at 7 days post-MCAO, infarct area is also reduced compared to WT (KbDb KO: 32% versus WT: 44%; $p = 0.03$) but to a lesser degree than in PirB KO. Together, these data suggest that knockout of PirB has a similar or even greater effect on infarct size than when Kb and Db are deleted.

To determine whether protection in PirB KO mice is also associated with improved motor performance, we assessed animals

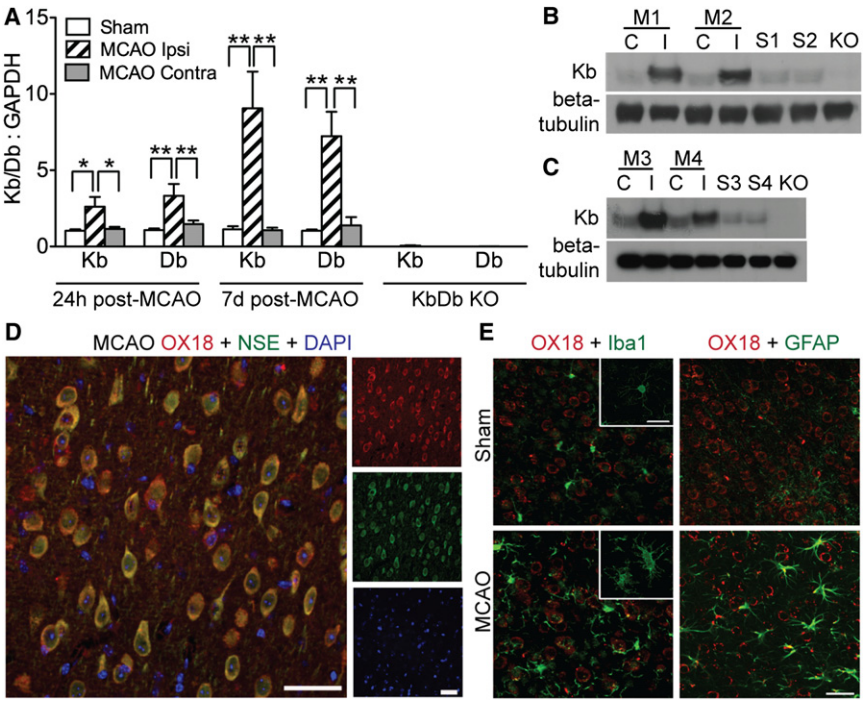

Figure 2. Expression of Kb and Db Increases in Brain of WT Mice after MCAO

(A) Kb and Db gene expression was measured using qPCR at 24 hr (n = 7 for each experimental condition) or 7 days (n = 7, MCAO; n = 4–8, sham) post-MCAO (ipsi, damaged hemisphere; contra, undamaged hemisphere). Negative control data from KbDb KO samples demonstrate primer specificity (Kb:GAPDH = 0.06 control relative to sham; Db:GAPDH = 0.01 control relative to sham; $p < 10^{-8}$ for both). *$p \leq 0.05$; **$p \leq 0.01$. (B and C) Western blots showing protein expression of Kb 7 days post-MCAO in synaptosome-enriched preparations (B) and in synaptoneurosomes (C). C, contralateral to injury; I, ipsilateral to injury; M1–4, different MCAO animals; S1–4, shams; KO, KbDb KO. (D and E) Immunohistochemistry of L5 in cortical penumbra using OX18 antibody. (D) Majority of MHCI immunostaining (OX18, red) co-localizes with neuronal marker NSE (green) 7 days post-MCAO. (E) GFAP (astrocytes, green) or Iba1 (microglia, green) 7 days post-MCAO. Scale bars in (D) and (E) represent 50 μm. qPCR data are represented as mean ± SEM. See also Figure S2.

on rotarod and foot fault. Prior to MCAO, KO mice learned faster and remained on the rod longer than WT over the course of the pretraining period ($p < 0.0001$; Figure S1E; no genotype differences were observed with training on the foot fault task, $p = 0.45$; Figure S1F). After MCAO, PirB KO mice performed better than WT on rotarod ($p = 0.001$) and foot fault ($p = 0.02$) by 7 days post treatment; even at 2 days post-MCAO, KO mice performed better than WT on foot fault ($p = 0.01$; Figures 3B and 3C). Together, these observations in PirB KO mice are strikingly similar to those for KbDb KO mice, suggesting that knocking out a receptor for these two MHCI molecules results in strong neuroprotection most apparent 7 days post-MCAO.

Astrocyte Activation Is Reduced after MCAO in PirB KO Mice

Experimentally and clinically, stroke is followed by an inflammatory response characterized by production of inflammatory cytokines, infiltration of leukocytes and monocytes, and activation of resident glial cells (Choe et al., 2011; Offner et al., 2006; Nedergaard and Dirnagl, 2005). Although activated astrocytes and microglia can exert beneficial effects, inflammation can also compromise neuronal survival and worsen ischemic damage. To determine whether PirB deletion alters glial activation 7 days post-MCAO, we immunolabeled brain sections for astrocyte and microglia and/or macrophage markers, and the number of activated cells in the cortical penumbra (Figure 3D) were counted. The number of reactive astrocytes decreased in PirB KO versus WT ($GFAP^+$: 26% decrease, $p = 0.001$; Figures 3E and 3F; $Vimentin^+$: 32% decrease, $p = 0.03$; Figures S3A and S3C). In contrast, the number of microglia did not differ from WT (Figures S3B and S3D). Thus, the neuroprotection afforded by PirB deletion appears to be accompanied by diminished numbers of activated astrocytes, but not microglia, in the penumbra area. A decrease in $Vimentin^+$ and $GFAP^+$ reactive astrocytes has been associated with better regenerative capacity after spinal cord or traumatic brain injury (Menet et al., 2003; Wilhelmsson et al., 2004). The diminished astrocytic, but not microglial, activation might reflect the contribution of astrocytes to synaptic plasticity and their close association to synapses (Beattie et al., 2002; reviewed in Giaume et al., 2010), where PirB and MHCI are thought to be located (Needleman et al., 2010; Shatz, 2009). Together, these observations suggest that the astrocytic response after MCAO relies in part on PirB signaling.

PirB Expression and Immediate Downstream Signaling Are Increased after MCAO

Because outcome is improved in PirB KO mice, we assessed whether PirB is upregulated in WT mice after MCAO. PirB protein levels are markedly increased in the damaged hemisphere post-MCAO, compared to the undamaged contralateral side or to sham controls (Figure 3G). Western blot analysis (input) verified the increase in Kb protein level in the damaged hemisphere (Figure 3H), similar to that observed in synaptosomes (Figure 2). In the damaged hemisphere, there is also a significant increase in β2m (Figure 3H). Because β2m is necessary for stable cell surface expression of the majority of MHCI proteins (Huh et al., 2000), it is likely that the increase in levels of Kb and Db is accompanied by increased cell-surface expression after MCAO.

MHCI binding to PirB facilitates tyrosine phosphorylation of PirB on cytoplasmic immunoreceptor tyrosine-based inhibitory motifs, which in turn recruits SHP-1 and SHP-2 phosphatases to PirB and modulates downstream signal transduction pathways (Nakamura et al., 2004; Takai, 2005). Therefore, we

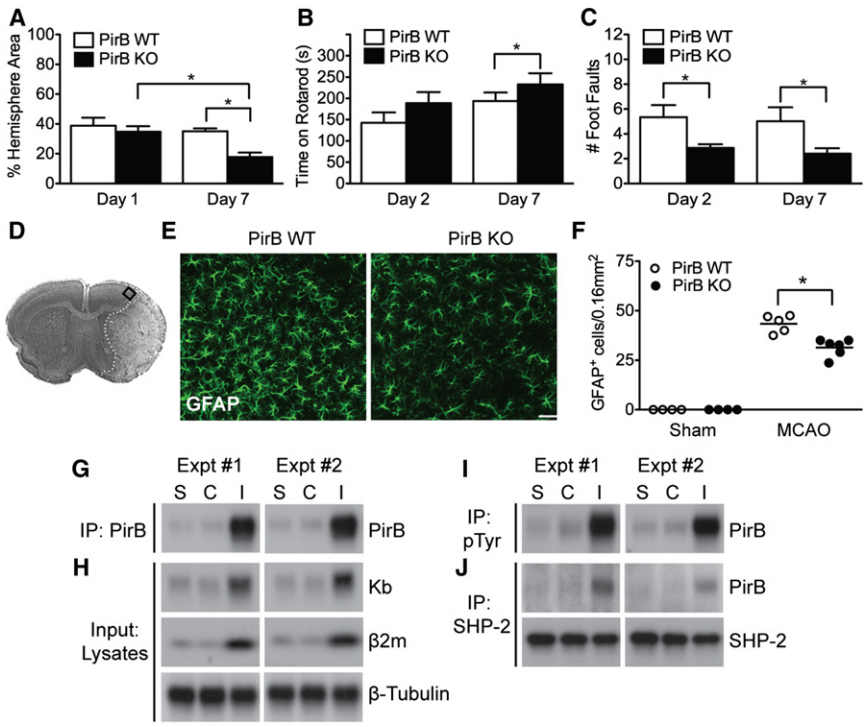

Figure 3. PirB KO Enhances Recovery and Dampens Astrocytic Response; MCAO Increases PirB Proximal Signaling

(A) KO animals have smaller infarct areas 7 days post-MCAO (n = 10 per genotype), but not 24 hr post-MCAO (n = 7 for WT; n = 8 for KO). Infarct area decreased 51% in KO mice at 7 days versus 1 day post-MCAO ($p = 0.003$). (B and C) KO animals outperform WT cohorts on motor tasks after MCAO. Average rotarod (B) and foot fault (C) performances at 2 days and 7 days post-MCAO. n = 10–20 animals per condition. Rotarod performance of PirB WT was not significantly different at 7 days compared to 2 days post-MCAO; $p = 0.61$. Data are represented as mean ± SEM. (D–F) Astroglial activation is diminished in cortical penumbra 7 days post-MCAO in PirB KO mice. (D) Cresyl-violet-stained section; rectangle indicates cortical area used for cell counting. (E) Representative images of astrocytes immunostained for GFAP at a distance of 400 μm from the ischemic core. (F) Quantitation of $GFAP^+$ cells in WT versus KO. *$p < 0.05$, n = 5 animals per experimental condition. Scale bars represent 50 μm. (G–J) Enhanced PirB expression and PirB proximal signaling after MCAO. (G) Protein extracts from 7 days post-MCAO (M) or sham (S) brains were immunoprecipitated with anti-PirB antibody, followed by western blotting for PirB. In each experiment (n = 2), four hemispheres from sham (S1–4; S5–8) or MCAO (M1–4; M5–8) brains were pooled for protein extraction. C, contralateral to injury; I, ipsilateral to injury. (H) Expression of Kb, β2m, and β-tubulin in brain lysates from (G). (I and J) Aliquots of protein extracts from (G) were subjected to immunoprecipitation for phosphotyrosine (I) or SHP-2 (J) to assess levels of tyrosine phosphorylation of PirB and SHP-2 recruitment to PirB. See also Figures S1 and S3.

examined whether upregulation of Kb, Db, β2m, and PirB after MCAO is associated with known PirB signaling components in the brain (Syken et al., 2006). Both PirB phosphorylation and SHP-2 recruitment to PirB increase significantly after MCAO (Figures 3I and 3J). Thus, a notable consequence of MCAO is to engage the first key steps in PirB downstream signal transduction.

Diminished Cell Death in PirB and KbDb KO in an In Vitro Model of Ischemia

PirB and KbDb KO mice have smaller infarcts and better motor recovery, suggesting that these molecules exert their deleterious effects in WT both by causing more cell death within the infarct and by limiting compensation via synaptic plasticity in surviving circuits. Because Kb, Db, and PirB also function in the immune system, the smaller infarcts seen in KO mice might arise from a dysregulated immune response (Maenaka and Jones, 1999; Takai, 2005), rather than from absence of expression in the CNS. To examine this possibility, we employed an in vitro model of ischemia: 15 min of oxygen glucose deprivation (OGD) of hippocampal organotypic slice cultures. The slices contain resident astrocytes and microglia but few if any peripheral immune cells. Circulating neutrophils, which might be present initially within these slices, have life spans of only 8–20 hr, and so are gone prior to experiments, which start after 2 weeks in vitro; no new peripheral immune cells can infiltrate in response to injury. The extent of neuronal cell death was assessed directly in CA1 by using propidium iodide (PI) immunofluorescence 24 hr after OGD insult (Ouyang et al., 2007; Figure 4A). Despite the absence of peripheral immune system infiltration, cultures from KbDb WT mice sustained significant damage, whereas cell death was significantly reduced in cultures from KbDb KO mice, as indicated by a 55% decrease in average PI fluorescence intensity (KbDb KO: 38 ± 1.9 median pixel intensity versus WT: 89 ± 3.4; $p < 0.0001$; Figure 4A). Cultures from PirB KO mice also had less cell death than WT, visualized as a 54% decrease in average PI fluorescence intensity compared to WT (PirB KO: 64 ± 3.7 median pixel intensity versus WT: 141 ± 7.3; $p < 0.0001$; Figure 4B). These observations demonstrate that in vitro as well as in vivo, PirB, Kb, and Db contribute to damage after ischemia. In addition, results suggest that, in vivo, the absence of these molecules in brain cells (neurons and/or resident glia), rather than just in the peripheral immune system, is neuroprotective.

Increased Crossed Projections of CST Axons after MCAO in PirB KO Mice

Functional recovery after stroke is associated with axonal plasticity as well as with altered gene expression profiles (Lee et al., 2004; Li et al., 2010; Netz et al., 1997; Stinear et al., 2007; reviewed in Benowitz and Carmichael, 2010). Cortical injury increases axonal projections descending from layer 5 (L5) pyramidal neurons in undamaged motor cortex that cross to innervate denervated subcortical targets, including red nucleus and spinal cord (Lee et al., 2004; Naus et al., 1985; Rouiller et al., 1991). L5 pyramidal neurons express PirB, and protein

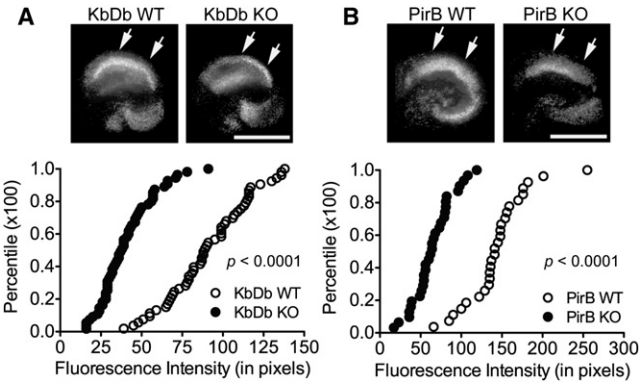

Figure 4. Diminished Neuronal Death in CA1 Region of Hippocampal Organotypic Slice Cultures from KO Mice after In Vitro Ischemia

PI-immunofluorescence (bright white) in CA1 region (top) and cumulative histograms of neuronal damage, as measured by fluorescence intensity at 24 hr after OGD (bottom), show that deletion of Kb and Db (A) or PirB (B) reduces cell death compared to WT. For cumulative histograms, each point is one slice (KbDb KO: n = 55 versus WT: n = 52 slices from five animals; PirB KO: n = 27 versus WT: n = 30 slices from four animals). Arrows indicate measured CA1 area. Scale bars represent 800 μm.

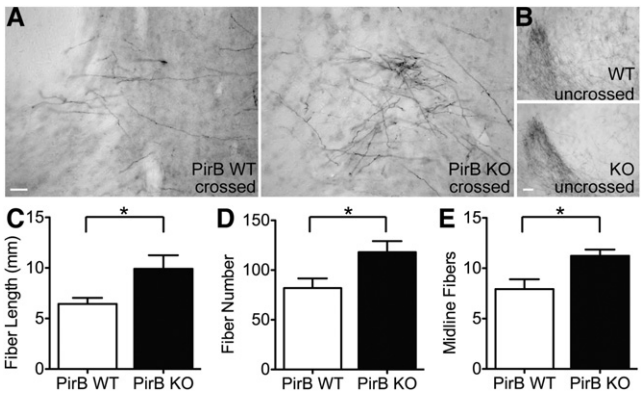

Figure 5. Increased Corticospinal Tract Fibers in PirB KO versus WT Mice 28 Days Post-MCAO

(A) Representative images of BDA-labeled crossed CST fibers originating in undamaged motor cortex and terminating in the red nucleus on the damaged side. (Midline is to the right of each image.) Note more extensive labeling in KO. (B) The uncrossed CST projection to the red nucleus terminating on the undamaged side. (Midline is to the left of each image.) Note similarity of labeling in KO versus WT. (C–E) Quantification of crossed fibers from undamaged CST by total fiber length (C) (WT = 6.5 ± 6.0 mm; KO = 9.9 ± 1.3 mm; p = 0.032), fiber number (D) (WT = 81.9 ± 9.7; KO = 118.1 ± 11.1; p = 0.036), or number of fibers crossing the midline (E) (WT = 7.9 ± 1.0; KO = 11.2 ± 0.6; p = 0.024). n = 5, KO; n = 6, WT; two to three sections per animal were analyzed. Scale bars represent 100 μm. Data are represented as mean ± SEM.

can be detected in descending corticofugal axon tracts during development, as well as in cortical neuron growth cones in vitro (Syken et al., 2006). Deletion of PirB increases axon outgrowth on myelin inhibitory substrates in vitro (Atwal et al., 2008). Consequently, it is possible that enhanced recovery from MCAO in PirB KO mice arises in part from an enhanced capacity of L5 pyramidal axons descending from the intact hemisphere to cross the midline into denervated territory.

To determine whether there are a greater number of crossed corticospinal tract (CST) fibers, we injected the anterograde tracer BDA into contralateral (undamaged hemisphere) motor cortex 14 days post-MCAO in PirB KO and WT to label the descending axons from L5 pyramidal neurons in the intact hemisphere. BDA-positive fibers were examined in the red nucleus ipsilateral (Figure 5A) or contralateral (Figure 5B) to the injury. In the ipsilateral red nucleus of PirB KO mice, there was an increase in all measured parameters of crossed axons: fiber length (Figure 5C; 52.3% increase in KO; p = 0.032), fiber number (Figure 5D; 44.2% increase in KO; p = 0.036), and the number of fibers crossing the midline (Figure 5E; 41.8% increase in KO; p = 0.024) were greater than in lesioned WT controls. To exclude the possibility that the increase in BDA-positive fibers was due to better labeling in KO than in WT mice, we calculated the mean pixel intensity of BDA labeling in contralateral red nucleus. No difference was seen between KO and WT (WT = 181.4 ± 3.1; KO = 175.8 ± 4.1; p = 0.30). The increase in labeled fibers in PirB KO mice is also unlikely to be due to a difference in infarct size, because average infarct index between WT and KO was not different at the conclusion of the tract-tracing experiment (WT index = 14.5 ± 6.6; KO index = 12.4 ± 5.3; p = 0.813). The increase in crossed CST fibers from the intact motor cortex that terminated within the denervated red nucleus in PirB KO mice could account for their improved behavioral outcome post-MCAO and suggests that L5 pyramidal neurons in these mice have greater axonal plasticity in response to stroke.

DISCUSSION

Here we show significant neuroprotection in the absence of either the innate immune receptor PirB or two of its MHCI ligands Kb and Db by using in vivo and in vitro ischemia models. Motor performance in KO mice recovered to a greater degree than in WT, and infarct area was smaller in KO but only after 7 days and not 24 hr post-MCAO. This delay is consistent with the idea that mechanisms of synaptic plasticity and functional recovery take time and may be more fully engaged in KO mice.

qRT-PCR and western blotting revealed dramatically increased expression levels of Kb and Db in the damaged hemisphere of WT mice. β2m protein, the light chain that is coexpressed with MHCI molecules (Zijlstra et al., 1990), is also elevated after MCAO, implying that there is an increase in stable cell-surface expression of MHCI protein. Because PirB expression, phosphorylation, and its interaction with SHP-2 are also increased, these observations argue mechanistically for an increase in signaling cascades downstream of the PirB receptor. Together, these experiments identify a set of molecules that, when present, exacerbate damage caused by stroke and, when removed, permit more extensive recovery.

The greater recovery in PirB versus KbDb KO mice fits well with a model in which PirB binds not only Kb and Db, but also other ligands. In addition to classical MHCIs, PirB is also thought to bind Nogo (Atwal et al., 2008) and to collaborate with the

Nogo receptor (NgR), which itself cannot signal (Fournier et al., 2002). Mice lacking Nogo or NgR, like PirB mice, have enhanced synaptic plasticity (McGee et al., 2005), and blocking NgR function also enhances recovery after MCAO (Lee et al., 2004). Thus, deletion of PirB would be expected to have a larger effect than deleting only a subset of ligands. It will be worthwhile to explore PirB interaction with other ligands as well as receptors in the context of neuroprotection from stroke. An important implication of the findings reported here is that new avenues of therapy after stroke may be available, because PirB in humans has only a limited number of homologs, members of the LILRB family (Takai, 2005). As a key step, it will be necessary to explore whether acute blockade of PirB or LILRBs can also lead to neuroprotection.

Increased CST Projection in PirB KO Can Account for Enhanced Functional Recovery

After stroke, neurons in undamaged cortical regions extend their axons into damaged regions and become responsive to motor or sensory functions perturbed by injury (Lee et al., 2004; Netz et al., 1997). In PirB KO mice, an increased number of midline crossing fibers from the undamaged corticospinal tract were seen extending into the denervated red nucleus 28 days post-MCAO. These observations support previous studies showing that PirB and MHCI ligands limit axonal outgrowth in development and regeneration after injury in vitro and in vivo (Atwal et al., 2008; Fujita et al., 2011; Washburn et al., 2011; Wu et al., 2011). In vivo, PirB downstream signaling inhibits Trk receptors that function to promote axonal outgrowth; KO of PirB increases TrkB signaling and neurite outgrowth after optic nerve injury (Fujita et al., 2011). However, our results contrast with recent studies that report no difference in PirB KO CST axonal projections using a traumatic brain or spinal cord injury model (Nakamura et al., 2011; Omoto et al., 2010). Note that these studies used entirely different injury paradigms as well as a different PirB KO mouse. Our study suggests that the increased number and length of CST fibers from the undamaged motor cortex that extend into the denervated red nucleus can serve as a structural substrate for the improved recovery observed in PirB KO mice after MCAO.

Is Protection Due to Changes in Immune Response, in Neural Plasticity, or in Both?

In the healthy brain, neurons express both MHCI and PirB, with MHCI protein detected at synapses (Datwani et al., 2009; Needleman et al., 2010; Figure 2). Genetic deletion of either Kb and Db or PirB results in enhanced synaptic plasticity in the visual cortex, hippocampus, and cerebellum in development and in adulthood (Datwani et al., 2009; Huh et al., 2000; McConnell et al., 2009; Syken et al., 2006), consistent with the proposal that MHCI and PirB receptor signaling limit synaptic plasticity in the healthy brain (Shatz, 2009). Thus, the significant elevation of MHCI and PirB expression, as well as PirB proximal signaling components after MCAO (Figures 2 and 3), could reduce synaptic plasticity of surviving neurons and circuits, thereby limiting functional recovery. Indeed, cellular correlates of synaptic plasticity, such as LTP, are blunted or absent after MCAO (Sopala et al., 2000; Wang et al., 2005). After MCAO, neurons are the chief cell type in the brain in which MHCI expression is upregulated, as identified by colocalization of the neuronal marker NSE with the OX18 antibody, which is known to recognize MHCIs in neurons and at synapses in rat and mouse (Datwani et al., 2009; Needleman et al., 2010; Neumann et al., 1995; Figure 2). An increase in Kb protein in synaptosomal preparations was also observed, consistent with the possibility that synaptic plasticity may be diminished after MCAO in WT mice. These biochemical preparations not only include pre- and postsynaptic membranes, but could also contain glial processes that enwrap synapses, so it is possible that upregulation also reflects a glial contribution. However, electron microscopy studies of MHCI protein in healthy brain sections show localization primarily at synaptic and subsynaptic neuronal membranes (Needleman et al., 2010), implying that neuronal MHCI can be upregulated.

MHCIs and PirB are also normally expressed in the peripheral immune system (Takai, 2005). KbDb KO mice have compromised adaptive immune systems due to dampened CD8 T cell responses (Schott et al., 2002). In contrast, PirB KO mice have intact, even hyperactive, adaptive immune systems (Nakamura et al., 2004). These diametrically opposed peripheral immune responses are not easily reconciled with the observations here that ablation of either PirB or MHCI leads to neuroprotection. The fact that these molecules are expressed and signal in neurons suggests that neuroprotection is at least in part brain specific. This conclusion is consistent with the OGD experiments using hippocampal slice cultures, prepared from the healthy brain, which lack functioning vasculature and in which peripheral immune cells cannot participate. Neurons in this model are thought to die as a consequence of apoptosis and necrosis (Meloni et al., 2011). The KO slice cultures suffered less neuronal cell death than WT cultures subjected to the same duration of OGD, consistent with the idea that the function of these molecules in the brain—in neurons or possibly other resident glial or microglia—normally contributes to damage after stroke. Future experiments involving cell-type-specific knockout mice will help dissect the relative contribution of peripheral immune cells, neurons, and brain glia to damage and impaired recovery.

EXPERIMENTAL PROCEDURES

Animals

KbDb KO mice, offspring of breeding pairs on a C57BL/6 background, were generously provided by H. Ploegh (Vugmeyster et al., 1998; Ziskin et al., 2007). C57BL/6 (i.e., KbDb WT) controls were purchased (Charles River). PirB KO and PirB WT controls were previously generated in C.J.S.'s laboratory (Syken et al., 2006). Mice were maintained in a pathogen-free environment. All experiments using animals were performed blind to genotype and in accordance with a protocol approved by the Stanford University Animal Care and Use Committee and in keeping with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Focal Cerebral Ischemia

Transient ischemia was induced in male mice (postnatal days 60–90) by using the suture occlusion technique, as previously described (Han et al., 2009), with slight modifications. This age was chosen because it is beyond the developmental critical periods, but the animals are still relatively young adults.

See Supplemental Experimental Procedures for complete details of experimental procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2012.01.020.

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